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THE PROPERTIES OF DESICCATED RABIES VIRUS AND ITS USE IN ANTIRABIC IMMUNIZATION.*†

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The preservation of rabies virus by desiccation has been reported by Harris and Shackell,[1] who, employing the method described by one of them (Shackell),[2] succeeded in maintaining the infectivity of this material for several months. The method consisted in freezing the brain or cord with a mixture of salt and ice and drying this *in vacuo* at a temperature below −10° C.

The object of this paper is to describe a modification of the original method, and to record the results of a quantitative determination of the amount of infectivity remaining in the dry material, and the value of this material in the study of the principles of antirabic immunization. I present also some observations on the essential difference between desiccation at the ordinary temperature and desiccation of thoroughly frozen material at a temperature below 0° C.

Tests of the material frozen by means of salt and ice as described in the first paper showed the amount of virulence remaining after complete desiccation to be from 1 to 2 per cent of the original quantity. Experiments undertaken to find a means of preserving a larger quantity of the infective agent resulted in the observation that *the more thoroughly and rapidly the material is frozen, the greater will be the amount of virulence remaining after desiccation.* When rabies virus is frozen with carbon dioxide snow and then dried *in vacuo* without being allowed to melt, from 30 to 50 per cent of the original infectivity is preserved. Allowing for a 60 to 70 per cent loss in weight, due to the extraction of the water, the dried cord contains as much virulence per milligram as the fresh.

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[1] *Jour. Infect. Dis.*, 1911, 8, p. 47.

[2] *Am. Jour. Physiol.*, 1909, 24, p. 325.

The method in detail is as follows: The brain or cord is ground in a porcelain mortar, with the addition of water, drop by drop, until a thick, smooth paste is formed. Carbon dioxide snow is then collected from a tank in the ordinary manner and is added in small amounts to the paste, which should be stirred thoroughly meanwhile to prevent the material from freezing in a solid mass. Freezing occurs rapidly and, when complete, the material is very brittle and easily reducible to a fine powder.

Carbon dioxide snow may be easily obtained from the ordinary tank of liquid carbon dioxide by allowing this to flow rapidly into a bag made of several layers of sterile towels. If the towels be wet beforehand the bag will freeze into a solid container and the snow may be emptied from time to time in any desired quantity.

During the pulverization more snow should be added from time to time to prevent thawing. When the material is thoroughly pulverized, it is transferred to a small beaker with an excess of snow and placed in the bottom of a Scheibler's vacuum jar which has previously been half immersed in a mixture of salt and ice and become thoroughly cold. A beaker of sulfuric acid is then placed on wire gauze in the upper part of the jar in such a manner that there is free access of air between the frozen material and the sulfuric acid. The acid is placed in the upper part because, if placed below, it soon freezes at the low temperature. The vacuum should measure less than 2 mm. of mercury.

During the desiccation the temperature should not be allowed to rise above -15° C. The jar should be rotated gently several times daily to mix the absorbed water and the acid. Unless this is done, the time required for the absorption of the water vapor by the acid will be unduly prolonged. A single brain will become thoroughly dry in from 36 to 48 hours. More material will require a longer time.

A satisfactory container for the ice and salt is easily made by surrounding a five-gallon earthenware jar with a thick layer of mineral wool. When this has once become cold throughout, the salt and ice will maintain a temperature below -15° C. for 36 to 48 hours. Should the amount of material to be dehydrated contain more water than will be readily absorbed by the sulfuric acid, the vacuum jar may be reopened and a fresh beaker of concentrated sulfuric acid changed for the diluted. During this transfer the temperature of the virus must not be allowed to rise and, to avoid this, the jar should remain semisubmerged in the cold mixture. In the same way a fresh supply of salt and ice may replace the melted brine, provided steps be taken to prevent a rise in the temperature of the jar meanwhile.

The object in thoroughly pulverizing the virus is twofold. It results in a more complete mixture, so that all parts contain an equal amount of virulence. Secondly, it permits of more rapid drying and an easy transfer into smaller containers for subsequent tests.

To avoid any absorption of moisture, it is preferable to transfer the dry powder from the beaker to small glass tubes and seal the end in the flame. This transfer is effected in a moisture-free atmosphere by covering the top of the beaker with rubber dam held in place by adhesive strips. A small puncture is made in this sheet large enough to admit the tube, and through this the tubes are inserted and filled. From 20 to 100 mg. is a convenient amount to put into each tube. If the diameter of the tube is 4 mm. each millimeter of powder will weigh approximately 2 mg.

A number of intracerebral injections were made with seven different lots of dried virus. In all of these the amount of desiccated *cord* necessary to produce paresis in a rabbit within six to seven days was not more than 1/50 of a milligram. The minimal lethal dose of desiccated *brain* is 1/200 of a milligram (0.000005 gm.). Tests were not made until the material had been thoroughly dry for at least seven days.

Although unknown to us at the time the first work was done, Vansteenbergh[1] had succeeded some years earlier in preserving the infectivity of rabies virus by desiccation *in vacuo*. He noted in 1903 that if rabic material were ground into a thin pulp, spread out in a *very thin layer* and desiccated in a rapidly produced vacuum, its infectivity could be preserved for many months, provided the resulting powder was kept in a dark, cool place, free from moisture. Others repeated this experiment, but agreed that the amount of virulence remaining was very small, and that the material was valueless for immunizing purposes (Stimson).[1]

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[1] *Compt. rend. Soc. de biol.*, 1903, 55, p. 1046.

Harvey and McKendrick[2] believe that the loss of virulence in cords dried by Pasteur's method is practically proportionate to the loss of water. In the first paper we stated: "Our work leads us to believe that it is the *method* of extracting the water which results in alteration or destruction of virulence, and not the extraction of water *per se*. To state it differently, slow desiccation attenuates and destroys the virus directly by reason of the concentration of salts and other substances which are in solution in the brain and cord. The action is therefore, in essence, a chemical one. Desiccation of frozen material avoids any concentration of those intra- and extracellular salts or substances which are at the ordinary temperature in solution. With this method absolute dryness proceeds cell by cell from the surface. If this proposition be true, Vansteenbergh's and Marie's successes and failures are easy of explanation. These authors emphasize the fact that the cord must be spread in a very thin layer and the vacuum produced very rapidly. A vacuum rapidly produced will freeze a small quantity of water in a bell jar. The success of these writers, in our opinion, depended upon the freezing of their thinly spread material and its drying without concentration. Further support is given to our hypothesis by the fact that exposure of thoroughly dried material to ordinary air destroys its virulence completely within a few hours. The absorbed atmospheric moisture is, in this case, sufficient to redissolve some of the salts and other soluble materials in a most concentrated state and destroy by chemical means the inclosed virus."

The present investigation has, it seems, established the correctness of the view expressed at that time. Partial freezing in the first work resulted in the conservation of only 1 or 2 per cent of virulence. Even the freezing with carbon dioxide snow does not result in complete solidification since the cryoscopic temperature is estimated to be between $-50°$ and $-100°$ C. But if this temperature (below $-50°$ C.) could be maintained until drying is complete, one should get a powder more virulent than that which

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[1] *Bull. No. 65*, Hygienic Lab., U.S. Public Health and Marine Hospital Service, Washington, 1910, p. 46.

[2] *Theory and Practice in Anti-rabic Immunization*, Calcutta, 1907.

I have secured by drying at −18° C. (salt and ice). Material frozen with liquid air and kept at that temperature until dry should contain practically all of the original infectivity.

To ascertain the physical effect of desiccation at low temperature, pieces of brain, kidney, liver, etc., have been frozen and dried *in toto*, fixed in absolute alcohol, transferred to xylol and imbedded in paraffin. These organs when frozen with salt and ice and dried at −18° show so much cell distortion as to be scarcely recognizable. When frozen with carbon dioxide and dried, as above described, the cells appear considerably altered and shriveled, with here and there an island on the surface fairly well preserved. When pieces of these organs are dropped into a beaker of liquid air and then dried, the sections show but slight morphological changes in the cells. Cell boundary and shape, nucleus and nucleolus are all preserved with surprisingly small changes. The alterations on the surface of the block are less than those in the deeper lying layers. These sections are striking illustrations of what small physical alterations occur in the drying of frozen material, and offer visible evidence of the extensive cellular alteration which results from osmosis and chemical change when dehydration is carried on at a temperature higher than the cryoscopic point.

Desiccated material kept in a cool, dark place (8 to 10°C.), free from moisture, loses its virulence very slowly. The following tests show the loss of virulence in one lot sealed and kept in an ice-box (8 to 10°C.).

R-184. Brains of three rabbits removed October 26. Placed on ice 24 hours, then ground up. Frozen with carbon dioxide snow and desiccated from October 27 to November 1, 1911. Transferred to tubes November 9 and sealed *in vacuo*.

November 10: 2 rabbits (A and B) inoculated with 0.02 mg.
2 " (C and D) " " 0.04 mg.
2 " (E and F) " " 0.20 mg.

Four of these (A, C, E, and F) were paretic on the 6th, one (D) on the 7th, and one (B) on the 8th day.

February 5: 1 rabbit inoculated with 0.05 mg.
1 " " " 0.10 mg.

The rabbits showed symptoms on the 6th and 7th day.

March 21 (1912) (142 days after complete desiccation):

1 rabbit inoculated with 0.10 mg.
1 " " " 1.0 mg.

Both rabbits paretic on the 7th day.

This material possesses, after a preservation *in vacuo* for 142 days, a virulence greater than that which Harvey and McKendrick found in two-day cords dried according to Pasteur's method.

Other lots of material kept in a desiccating jar in the presence of sulfuric acid show the same rate of loss in infectivity as when kept in sealed tubes. When kept in the light or in the presence of phosphoric anhydride the virulence is lost more rapidly. The loss at room temperature has not as yet been determined.

Liquid air has been used as a preliminary freezing medium, and while the desiccated powder was slightly more virulent (M.L.D. of brain = 0.000004 gm.) the results are of more scientific interest than practical value.

Tests of the immunizing properties of this material have been made on dogs, rabbits, and human beings. Several dogs, which had received severe lacerations and deep bites about the head by dogs known to be rabid, have been immunized and deep injections of a large amount of rabid dog's brain have been given to immunized dogs. The number of immunizing doses has varied from four to seven and the amount injected has varied from 10 to 50 mg. per dose. None of these animals has shown symptoms of rabies. Some were killed after one to two months and their brains found to be non-infective.

About 50 rabbits have been immunized and tested. These received from five to 14 immunizing doses of from 10 to 30 mg. each. In no instance has the injection of this material produced symptoms of rabies either in dogs or rabbits. Some of the rabbits have shown a partial immunization to subsequent subdural injection of street virus, and some have shown a complete immunity to intraocular injections of street virus.

In those that died, the period of incubation of the disease was lengthened from 18 days (control) to 40 and 60 days. Inasmuch as all investigators are agreed that complete immunity is established in the rabbit only after a prolonged treatment, I did not anticipate that these animals would survive the tests. The object of these tests was more to establish the innocuousness of the subcutaneous injections of the material than to produce a complete immunity in rabbits.

There are at the present time a number of dogs and rabbits under observation, and when these and other tests have been completed a subsequent paper will give a detailed and tabulated result of this part of the work.

The injections into human beings were not made until it had been shown that as many as 1,000 to 3,000 times a dose fatal to a rabbit (intracerebral) could be injected subcutaneously into dogs and rabbits without ill result. The first to receive injections was one of the laboratory staff. He received seven daily injections each of which contained 1,000 times the infective dose for rabbits. Since that time the material has been used in the treatment of 17 persons bitten by rabid dogs and cats.

This brings me to a discussion of the value of this material in settling some of the differences of opinion and practice in antirabic treatment. In some institutes treatment is begun with 14- or 10-, or 6- or 5-day cord. Some use Högyes' method, others follow the method of Ferran. There are in fact almost as many modifications as there are antirabic institutes.

Nitsch and others have shown that the amount of infective material varies throughout the cord, and that the cervical region is five times as virulent as the lumbar. It is evident from this that the amount of infective material, or, to use a more exact term, the number of lethal rabbit doses, injected from day to day is variable and does not depend entirely upon the amount of cord used. Even with the so-called exact method of Högyes, the variation must be considerable unless the same portion of the cord is chosen from day to day.

If the conclusion of Harvey and McKendrick that "the immunizing power of a given portion of rabies cord is a function of the unkilled remnant of rabies virus which is contained in that cord," is true, one should be able to find out with mathematical certainty how many minimum infective doses will produce a definite degree of immunity.

The very gradual and constant rate with which infectivity diminishes in desiccated material enables one to ascertain the number of M.L.D. administered in each injection. In these experiments I have adopted as a standardizing unit the smallest quantity which, when injected intracerebrally in a full grown rabbit, will produce paresis within six or seven days. In determining the degree of immunity established, it is also necessary to know the exact amount of street virus injected. For this purpose the infectivity of a desiccated brain (street virus) is being standardized.

The following problems present themselves in this connection and are offered here in the hope that others may be induced to work along this line.

To what extent does immunization depend upon the number of infective units injected?

Are a few large doses as efficient as the large number of injections of varying strength given according to the methods of Pasteur and Högyes?

Is there any danger in commencing the treatment with large doses, e.g., 1,000 units, and repeating this daily?

Have all strains of fixed virus approximately the same virulence?

Are all strains equally innocuous to dogs and rabbits and human beings when administered subcutaneously in the usual manner of treatment?

The questions above outlined are by no means new. They have, in fact, been asked many times before. I repeat them here, however, because I believe they may be more easily decided by the use of a virus of known and relatively permanent infectivity, i.e., a standardized virus.

SUMMARY.

Rabic material may be completely desiccated without destruction of virulence, provided the dehydration takes place at a low temperature.

The lower the temperature, the greater will be the amount of virulence preserved.

The desiccated virus contains per weight as much infectivity as the fresh virus.

The loss of virulence is so slow that the material may be standardized, permitting an accuracy of dosage hitherto impossible.

The unit is the smallest amount which, when injected intra-

cerebrally into a full grown rabbit, will produce paresis on the seventh day.

The use of desiccated virus in antirabic immunization of animals and persons offers many advantages over other methods.

I wish to express my obligation to Mr. L. F. Shackell, whose enthusiasm led to the undertaking of the problem, and whose advice has been of much assistance in working out the details of the present method.